IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Westbrook	§	
	§	Examiner: J. Fredman
Serial No.: 07/784,222	§	
	§	Group Art Unit: 1634
Filed: October 28, 1991	§	
	§	Atty. Dkt: ARCD-010/NAK
For: METHODS AND COMPOSITIONS FOR THE DETECTION OF CHROMOSOMAL ABERRATIONS	§	

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

Sept 1, 1998 Richard A. Nakashima
Date

DECLARATION UNDER RULE 131

I, CAROL A. WESTBROOK, HEREBY DECLARE AS FOLLOWS:

1. I am the named inventor of the subject matter claimed in the referenced U.S. patent application, Serial No. 07/784,222, filed October 28, 1991.

2. I understand that the Patent and Trademark Office Examiner in charge of examining this application has cited against my application the following publication:

Tkachuk *et al.*, "Detection of *bcr-abl* Fusion in Chronic Myelogeneous Leukemia by in Situ Hybridization," *Science* 250: 559-562, 1990.

3. The invention of claims 1-3 and 5-35 was made and tested in the United States prior to October 26, 1990, and therefore prior to publication of the cited article by Tkachuk *et al.*

4. The fact that the invention of claims 1-3 and 5-35 was made and tested in this country prior to October 26, 1990 is evidenced by studies set forth in the attached notebook extracts (Exhibit A). Among other things, this Exhibit sets forth the following studies which exemplify the practice of my invention:

a) Possession and use of the c-Hu-ABL, PEM12 and MSB-1 probes in *in situ* hybridization experiments for detection of chromosomal aberrations in leukemic cell lines and in blood cells from patients with leukemia (Pages 1-2 and 5-43 of Exhibit A).

b) Identification of doublets in the chromosomal DNA of leukemic cell lines and blood cells from patients with leukemia using distinguishably labeled probes specific for the c-H-*abl* and *bcr* genes. (Pages 1-2, 5-6 and 21-24 of Exhibit A).

c) A detailed protocol for detection of the c-H-*abl/bcr* fusion gene, using distinguishably labeled probes specific for the c-H-*abl* and *bcr* genes. (Pages 5, 7-11, 13-22, 24, 26-31, 33-43 of Exhibit A).

Each of items a) through c) as represented in the attached Exhibit were carried out in this country prior to October 26, 1990.

5. All statements made in this Declaration of my own knowledge are true and all statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001 and may jeopardize the validity of this application or any patent issuing thereon.

Aug 3 '98
Date

Carol A. Westbrook
Carol A. Westbrook

Results:

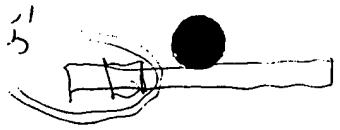
(1) MH5.15 on ALH:
Cells "fuzzy" – lot of cytoplasm
no signal detected.

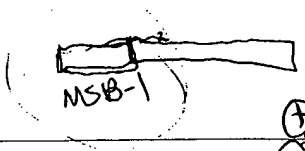
* Problem is ē slides? Too much cyto, poor penetration of probe despite Proteinase Treatment.
∴ Will discard slides which look like this one – make new control slides.

(2) Abl alone on SupB13
PERFECT! Clean slides, consistent signal most ē 2 dots/nucl.

(3) MSB-1 alone on SupB13
fair results – many nuclei ē consistent signal but background high

(4) Double hybs:
SupB13 – good results - clear consistent signal but problem of timing –
Red signals seen first, fade quickly as they fade green signals come up
Many doublets clearly visualized: red-green side by s
Red background still high.
ALH – many double signals, no doublets
slide less satisfactory than other (SupB13)





PEB (+)
P1.90 (+)
(+) on PEGE
(-) non So. BCR

Conclusions
- Amplification step works ≤ difficulty, + no obvious cross reactivity.
- MSB - shows good specific hyd'zn
- Doublets obvious
- Need the computer imager! OR More amplification to get photos

Mon: Will prepare new slides:
- controls
- Sup B 13
- patient slides — have 3 patients & ALL from CALGB study
-

Work out steps / order reagents for another round of amplification

Make 10% BSA solns to mix for blocking
10% SSA

Mon

- Fluorescence + imaging back from library...
- Flow cytometry paper - ? nuclear suspension preparation
- Dropped 4 sets of slides:
  - JK control slides -
  - J Smith - peripheral blood ALL
  - P Orzell - bone marrow ALL
  - Davis - bone marrow, frozen ALL

20 - 25 slides each, still some cells in fix 4°.
Rare mature PMNs or bands, but present in Smith + Orzell slides good quality
Davis - poor cell "quality" Freezing artifact apparent.

- Thawed cells from Varga, Anne ALL Ph^+, bcr^-
  to test 50×10^6 cells
  Incubated ½ o/n
  " ½ 3° to allow cells to recover from freezing

over

- Dr Grieme & Susan McKibben - senior student
5-5723
Discussed objective of my analyses at length -
trial run Thurs. AM.

Weds: Worked on Grant application...

Thurs AM: Dr. Grieme & Susan McKibben 5-5723
Took 319-scope down to lab -
images captured fuzzy, had problems ā focus
used double-hyb-d SUPB13 from

Also, amount of light camera is sensitive too may be
too much for the fluorescent assay?
too much "junk"?

Uncertain, but worth pursuing at least until set-up
for fluorescence is perfected - then decide.

Thurs PM -
Harvested cells from Tues. - from Varga-PB
Good yield - did metaphase harvest.

Thurs PM

Hybridize: Patient Varga: MSB-1, cHu-abl, + double SuPB13 as control - double

① Ppt of DNA:

Exp't DNA	MSB	ABL	Plac DNA	SS DNA	KOAc	EtOH	Dextr/Form
MSB bsl.	√15	-	1	1	2	200	5/5
Abl bsl.	-	√5	1	1	1	160	5/5
MSB/ABL 2d	√30	√10	2	2	5	500	10/10

Used "new" MSB-1

Abl - almost gone

Into -70° x 30° - spin, dry, reconst.

5' at +70° - into 37°

② ~~Slide prep~~.

✓① Slides on warmer - 1½°

✓② RNAse 1° 37°

③ Wash x2 2xSSC 2'

✓④ Dehydrate & dry

✓⑤ Formamide 70% 70° 2'

✓⑥ Dehydrate & dry

✓⑦ Proteinase K 7.5' 37°

⑧ Dehydrate & dry

⑨ Hybridize - 90° x2'

⑩ 37° O/N.

Results:

Specific signals seen on all, c.
many doublets
High background

C&W suggests the following:

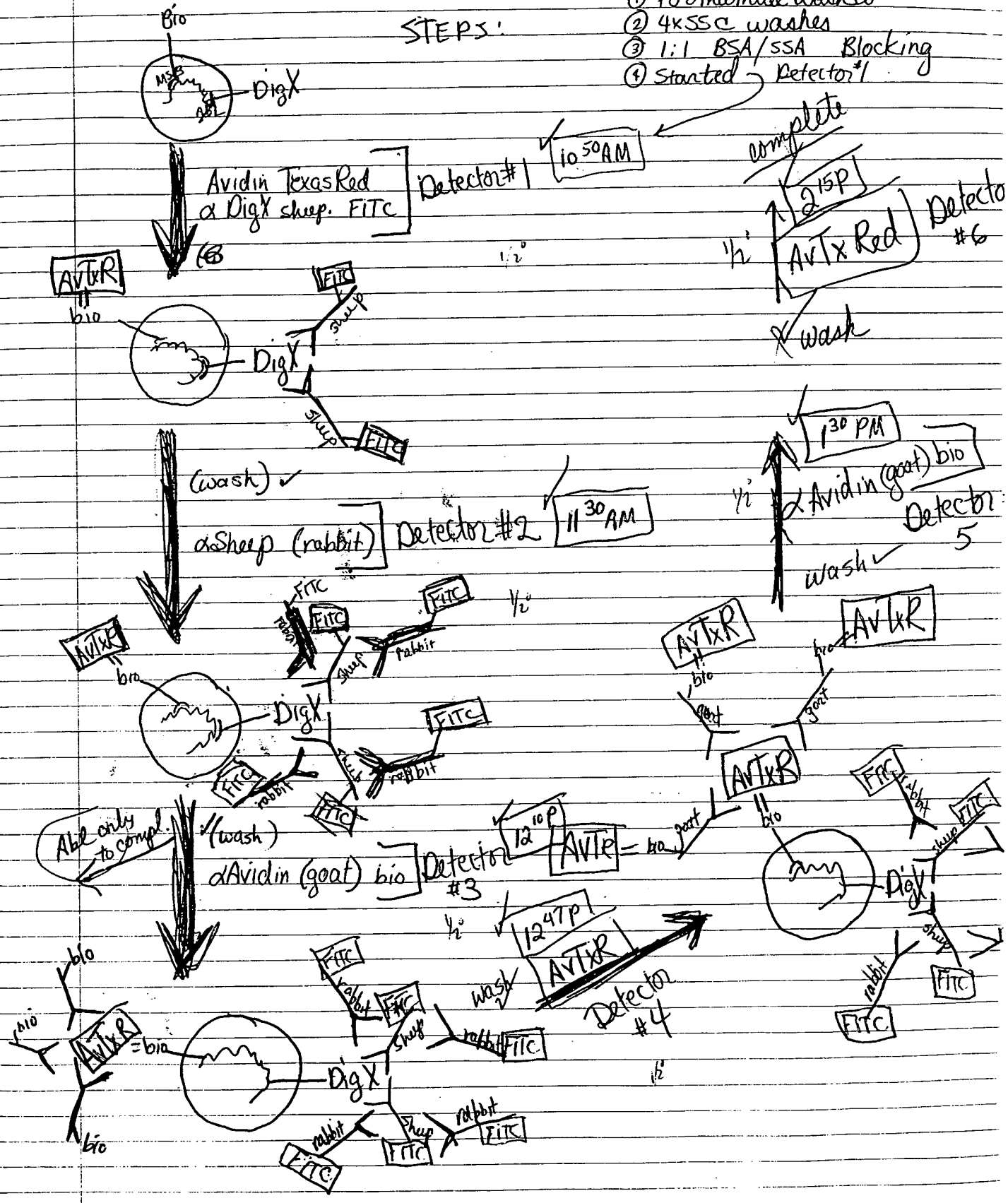
① Try c one color only; technical aspects of 2-color too much for most labs

②

FRIDAY : Detection Day ...

STEPS:

① Formamide washes
② 4xSSC washes
③ 1:1 BSA/SSA Blocking
④ Started → Detector #1



Detector #1: Avidin TxRed] in Sheep alb. 3%
αDigX]
for 2 slides: 600 →

✓400 µl 4x/Triton
✓200 µl SSA 3%
20 λ AvTxR
20 λ αDigX

For one Slide each:

Avidin TexR – see below
αDigX ← ~~400 µl~~ 375 µl :

✓256
~~400~~ µl 4x Triton
~~200~~ µl ~~5~~SA
✓125
10 µl αDigX Fluorescein

Avidin TexR for: MSB-only Slide 200
Det. # 4 3 Slides ~~8~~ 600
6 3 Slides ~~8~~ 600
+ 200
1600 µl
↓
1800

✓1200 µl 4x Triton
✓600 µl ~~5~~SA
70 µl AvTxR

αAvidin-bio (goat) ~~AASB~~ Detector #3 3 slides 600
Detector #5 3 slides 600
200
1400 → 1500

✓1000 4x/triton
✓500 3% SSA
60 µl Ab

αSheep (rabbit) Detector # 2 3 Slides 600 µl
↓
900

✓600 µl 4x/triton
✓300 µl B
30 µ Ab

DigX-label probes: PEM12, MSB1, KK5.33 (cosmid 5)

* Nick translate kits "pilfered". No stop Buffer, no reaction buffer left, vials were mixed between the two kits... will try as no new kits available → ordered thru

DNase dil –

1:10 – 1 in 9

1:100 1 in 9 of 1:10

1:500 2 in 8 of 1:100

	Expt'l DNA 1µg	10×RB	dNTP's	DigX-dUTP	water	1:500 DNase DI
MSB1	√4 use 8 next time	√5	√5	√5	23	4
PEM12	√7.5 use 4 next time	√5	√5	√5	19.5	4
KK5.33	√10 use 5 next time	√5	5	5	17	4

Combined – 14° ×2°

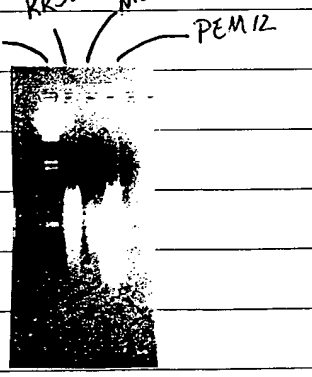
Made 200mM EDTA: 20 µl 500 mM EDTA
30 µl H2O

5 × " " + 65° × 10'

Ran out on gel:

KK5.33 + MSB1 need more cutting; PEM12 OK

(? MSB concentration less & PEM12 more than ~~out~~ calculated by O.D. reading?)



For AM:

(1) Will add MgAc (2 × 1M) to MSB + KK5.33 tubes & re-run them tomorrow when I nick-translate more MSB-1 & PEM-12 & bio-dUTP.

(2) Test DNase of new kit – old one running out.

(3) order 2 new nick-transl. kits & HIDE THEM! (Enzo LK

(4) " more digoxigenin-dUTP (Boehringer Mannheim 1093 08

(5) order meshes for soft tissue work. $100 for 25 nm

Ti

I Re-do nick transl. of [redacted]sB + KK5.33
II Test DNase dil. of new nick transl. kits
III Ppt DNA for a hybr. run
IV. Hybr. 6 slides - single color.
V. Make more RNAse

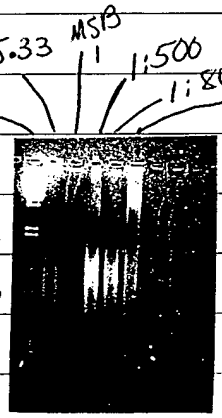
I. Re-start nick-trans. for MSB-1 + KK5.33 - dig'l

New dilution 1:500 DNase -

1 λ into 9 DB = 1:10
1 1:10 into 9 DB = 1:100
2 into 8 = 1:500 ✓

Add 4 λ DNase
4 λ DNa Pol.
2 λ 1 M Mg Ac
} to each tube

Will ✓ p 1°



KK 5.3 = not OK
MSB - ? little DNA
New DNAs 1:500 a

* out of DNA pol. from BOTH KITS!

~~Probably cause of p~~

II. Test dilutions for new DNase : 1:500, 1:800, 1:1000

1:10 = 1 in 9 — 1 of 1:10 into 9 = 1:100
1 of 1:100 into 4 = 1:500
1 of " into 7 = 1:800
1 " " into 9 = 1:1000

	Control DNa	RB	TTP	dNTP	H_2O	DNase	DNa pol
1:500	✓4	✓5	5	✓5	13	4	4
1:800	✓4	✓5	5	✓5	13	4	4
1:1000	✓4	✓5	5	✓5	13	4	4

Stop p 2°

III. Make more stock RNAse → 10 5ml aliquots.

IV Test digk-labeled probes from
PEM 12 + ~~MSB~~ MSB-1
and do double-labeling & single color

Sup B13 ~~X~~ MSB-1
2. MSB-1 / PEM 12 - 2 slides
3. MSB-1 / ~~PEM 12~~ / ABL - 2 slides

· BV 173 1. PEM-12
2. MSB-1 / PEM-12
3. MSB-1 / PEM 12 / ABL

~~MSB~~ ABL removed

	DNA MSB	DNA PEM	DNA ABL	PlacDNA	ssDNA	KoAc	EtOH	Dextr. Form.
~~MSB~~ (1 slide)	15	-	-	1	1	2	200	5/5
PEM-12 (1 slide)	-	√15	-	1	1	2	200	5/5
MSB/PEM (11 slides)	√15	√15	-	1	1	3	300	10/10
MSB/PEM/ABL (11 slides)	√15	√15	5	1	1	4	400	10/10

Combined - freeg - ppt - reconst

1. Slides on warmer
2. into RNase 1°
3. 4x wash 2x SSC 2'
4. Dehydrate + dry : 3'
5. 70% formd 70° 2'
6. Dehydrate + dry
7. Proteinase K 7.5'
8. Dehydrate + dry
9. Hybridize + seal
90° 2' → 37° O/N

Nick translation results:
* PEM 12 OK but a lot of DNA - (will begin hyb's @ 10 λ instead of 15 λ)

* MSB-1 very little - next nick trans. will double a

* KK 5.33 still didn't cut well
* DNase dil for 2nd kit atleast 1:500

Weds:

✓Make 4xSSC / 0.1% Triton
✓Make 50% formamide / 4xSSC ⟶ into water bath
Make 4xSSC
Lab meeting!
Dev. Slides:—
c one round amplification.

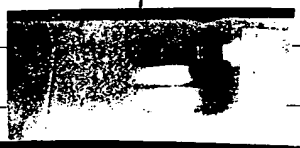
No major problems.

Thurs. — Guest lecturer
✓Worked on proposal

FR

New nick translation kits arrived:
Taped one shut, ē my name + into freezer. (Labe
Other one "A" - will test DNase dilutions
1:500, 1:1000 + 1:1800
1 in 9 → 1:10 → 1 in 9 = 1:100
1 in 4 = 1:500
1 in 9 = 1:1000
1 in 11 = 1:1200

	Rxn Buff	dNTP	TTP	↓ Control DNA	H_2O	DNase	DNA Pol
1:500	√5	√5	√4	√4	√24	4	4
1:1000	√5	√5	√4	√4	√24	4	4



added Mg Ac to rxn as our DNA is dissolve in TE - may be part of problem ē past Nick-trans's. Didn't add enough at 1st, so ↓ 2° of rxn, ran gel ē some of reaction mix, added more Mg++ to rest, ran x1' more.

Mon -
Will use 1:500:
Nick translate MSB-1 ē digX - will use more this
PEM 12 ē digX
Abl ē biotin
KK5.33 ē digX

Exp. Diff	MgOAc 500mM	Rxn Buffer	bio-dUTP	digX	dNTP	H_2O	1:500 DNAse	DN
MSB	√6 λ	2	√5	0	√5	√5	19	4	
PEM	N7 λ	2	√5	0	√5	√5	~~20~~ 18	4	
ABL	√5 λ	2	√5	√0.5		√5	20	4	
~~MSB~~	√2 λ	2	√5	0	√5	√5	23	4	

over

Dilution DNAse
1:10 1 in 9
Gl in 9 = 1:100
4 in 16 = 1:500

2^{45} – 4^{45} Reaction Run.
5λ Stop buffer 10' heating block
Will run gel in early AM. & gene-clean

~~Make reagents~~ ~~of~~
In AM:
① Hybridize patient slides – direct preps from ALL, Katsikas –
using probes nick-translated today if they are OK on gel.
② Make reagents – 4xSSC, 2xSSC, @ 4xSSC/0.1% Triton
③ Prepare MSB-1 drag DNA for shipping ā note.

④ Cells from Marina? and name of Houston doc (8p bmbs

Worked on Am Ca Society Grant Proposal

Turned in / Fed Ex'd FRI 5PM.

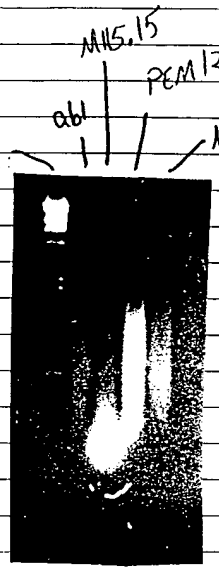
Range of probes nick-translated last week.

Only MH5.15 did well, a lot of DNA used 2λ

MSB-1: about right ~~Still a light~~ on the DNA used 6λ ~~(not enough) but better~~ (too much)

PEM12 still heavy

Problem c Abl – ? don't know



Plan: 1. Restart rxns of Abl, MSB, PEM12
2. Concurrently run New ones – will need lots of probe for ALL study
still need digX – PEM & MSB

For the "re start" –

	MgOAc ~~250~~ mM	DNA	dNTP	digX	biodUTP	1:500 DNAse	DNA pol
Abl	~~1~~	1λ	2	~~Ø~~	2	4	4
PEM12	~~1~~	Ø		2	Ø	4	4
MSB-1	~~1~~	1λ	2	2	Ø	4	4

total vol ~54 λ

Need to add MgAC to 5mM in 50λ
would be 1λ of a 250mM solution

~~250~~ mM = (.250 M)(15 ml) = (1 M MgAc)stock x X
3.75 ml = X

3.75 ml	MgAC
11.25 ml	H_2O

ON HOLD per CAW
will test them on the n cells

For the "fresh start" in AM

	Exp DNA	RxnBuff	dNTP	250mM MgAc	biodUTP	digX	H_2O	1:500 DNAse	DNA pol
Abl	7λ	5	5	1	5	Ø	19	4	4
MSB-1	6λ	5	5	1	Ø	5	21	4	4
PEM12	5λ	5	5	1	Ø	5	21	4	4

~~Need 24 λ of DNAse did~~ HALF ONE

1 in 4
2 in 8
4 in 16
~~6 in 24~~
8 in 3

DNAse dilution
1 in 9 = 1:10
1 of 1:10 in 9 = 1:100

1 in 4 → 4 in 16
5

5 of 1:100 into 20

~~Start AM~~

CAW suggests withholding DNA pol. until DNase is done — will try & run in AM

On the meantime: Will run gel (0.8%) to ✓ concentration/purity of 8/2/90 phage prep. with both Hind III cut Øλ and uncut Øλ

3 λ PEM 12

6 λ MSB

1 µg uncut Øλ

1 µg HinDIII Øλ

Conclusion:

CME 9 4 2 ~~7699~~
Vicki OSull 7119

	Exp. DNA	RxnB.	dNTP	bioUTP	DigUTP	H_2O	1:500 DNAse
Abl	✓5	✓5	✓5	✓5	~~✓5~~	22	4
MSB-1	✓6	✓5	✓5	-	✓5	21	4
PEM-12	✓3	✓5	✓5	-	✓5	24	4

↳ not enough. use 4λ

Reaction started: 9[33] AM. → 14° Ran 1° 10'
Will Add 4λ DNA pol. at 10[43] A.
Added DNA pol at 11[02] A, ran 1°, heated to 65° x10'
returned to 14° x1 more hour.
Added 5λ 200 mM EDTA. to stop.
DNAse dil: 1 in 9 = 1:10
1 (of 1:10) in 9 = 1:100
3 in 12 = 1:500 for 3 tubes

Ran on minigel to ✓ for cutting:

Conclusions: ① cutting good!
② Amt of MSB-1 (6λ) is good of PEM-12 (3λ) not enough. Will use 4λ next time.

For Thurs: (Weds. is Fannie May/Coleman Cancer Confc.
① hybridize six normal slides, comparing the probes from nick translation to those of today.
② get frozen tumor from Lori; try collagenase digestion.

I. Hybridize 6 nl slides to compare probes ~ 500 to 800 c̄ probes < 500

		Exp'l DNA	PlacDNA	ssDNA	KOAc	EtOH	Formd/DexSulf.
Abl	10/1	5	1	1	1	100	5/5
Abl	10/2	5	1	1	1	100	5/5
MSB-1	10/1	15	1	1	1.5	200	5/5
MSB-1	10/2	15	1	1	1.5	200	5/5
PEM12	10/1	15	1	1	1.5	200	5/5
PEM12	10/2	15	1	1	1.5	200	5/5

Precipitated DNA for each as above.
Six ALH 8/13 slides thru day #1 protocol; fresh reagents, no problems.

II. Tumor digestion:
- Collagenase diluted to 2000 u/ml, aliquots of 1 ml frozen (used 10 mg of collagenase)
- Tumor #90- from Lori, c̄ ~5g + ~18g
- 4.5 ml media + minced tumor + 0.5 ml collagenase
- O/n incubation.

For AM:
① Develop slides, c̄ one amplification step
② ✓ colon tumor digestion

I. Colon tumor digestion

Transferred cells + media + collagenase to tube — allowed lg fragments to settle Removed cells in suspension, returned large pieces of tissue to flask ē more collagenase

Spun suspended cells — ? hopefully single cells
Hypotonic KCL + fix 3:1
Dropped a test slide → Bacteria! No ep. cells

This approach won't work — ? fix first then digest
Need to find new protocol

II. Detection steps for hybridized slides:
50% formamide x3, 4xSSC x3, serum block x1°, then:

A. For biotin-Abl slides

Avidin Texas Red in 3% BSA / 4x/5% Triton X 200/40l

amplified ē —

10 λ bio-α-Avidin (in goat) in: 400 λ 4xSSC/Triton X
200 λ 3% SSA

+ another round of Avidin Texas Red as above

B. For DigX-MSB1 + DigX-PEM-2

15 λ α digX (in sheep) in 800 4xSSC/TritX
400 3% SSA

then amplify:

FITC-α-sheep (in rabbit) in 800
400

Washed 3' x2 in 4xSSC/.1% Triton X between 1° + amplification steps.

Results -

All slides show signal: bright, clear, specific

PEM 12 — both excellent

MSB-1 — = OK but usable

MSB-1 — bottle

Abl — OK but

Slides hazy. Problem ā BSA?

background high in FITC/Dig slides

- may need to add rabbit serum to blocking step
- Wash x3 in between 1° Ab + amplification step

Conclusions:

① All probes usable. Size difference seems to be important for MSB-1 only, & then only a little

② Need work on dig background

FOR NEXT WEEK:

① Hybridize patient slides

② " CML colony cells from Toronto

③ Try again ā another disaggregation protocol

	PEM12	MSB-1	ABL	PlacDNA	ssDNA	KOAC	EtOH	Form/deg
PEM/Abl x5	60λ	–	25λ	5	3	9	300	25/2
MSB/ABL x3	~~45λ~~	45λ	15λ	3	2	6.5	200	15/15

MSB 15/slide
PEM 12/slide
Ppt + reconstitute DNA.

Slides to be done: (8)

BV 173 : Control for PEM-12 Abl x1
Toronto CML Slide : PEM12-Abl x2
JSmith : ALL . PB (unknown) : " " x1
} 5 PEM 12-ABL

SUP B13 control for MSB-Abl x1
JSmith ALL-PB (unknown) MSB-Abl x1
Varga ALL-PB (known) MSB-Abl x1
} 3 MSB-Abl

Varga ALL-PB (known) PEM12-Abl x1

1. Slide warm: (5° X 4°
2. RNAse 37° X 1°
3. 2x SSC x4 2'
4. Dehydrate + dry
5. 70% formamide/4xSSC x2' 70°
6. Dehydrate + dry
7. Proteinase K
8. Dehydrate + dry
9. Hybridize + seal
10. 90° x2', 37° O/N

For tomorrow :- Slide detection steps
- Prepare journal club
- Talk to Tony Montag re: slides (if any);
CL cond & lunch to discuss tissue Dx

I. Detection steps

✓1. Formamide 50% wash x3 5'

✓2. 4xSSC washes

✓3. Blocking 5% BSA/SSA/3% Rabbit 10A

✓4. Detector #1 (see 14 Sept 90)

AvTxRed) for 8 slides : 1600 + 200 for filter = 1800
Biotin)

1200 4xSSC/Trit)
600 SSA)

~10µl/ml (20µl λ AVTexR
20µl λ αDigX

✓ wash x3

✓5. Detector #2

α sheep in rabbit serum : 1200 4xSSC/Trit
600 ~~5%~~ Rabbit serum,
20λ ~~α~~ Sheep

✓ wash x3

✓6. Detector #3

bio-αAvidin (goat) in sheep 1200 4x/Trit
600 SSA
20λ αAv

✓ wash x3

✓7. Detector #4 Avidin Tex Red 1200 4xSSC/Trit
600 SSA
20λ AV TxR

Wash x3

~~X~~ 8. DAPI x3'

9. Wash 4xSSC x1

10. DAPCO/coverslip

Will examine slides in AM,
do counts
take photos.

cont'd

Cells were intact, unlike Varga who had been frozen. Preparation fairly clean, moderate background.

3. Positive control slides. SupB13-MSB/ABL had very yellow nuclear background staining, poor hybridization and no doublets seen. Bad prep for unclear reasons. But the high yellow nuclear background is similar to the first succussful run - may be a property of the cell line, or may need different treatment. Will think it over!

BV173-PEM/ABL with good hybridization, little background, multiple clear doublets in the majority of cells.

"RULES OF THE ROAD" FOR INTERPRETING AND COUNTING PREPS:

1. Count only intact nuclei with clearly visible rim. Skip smudged, partly smashed, or fractured nuclei.
2. Count only nuclei which have instantly obvious red and green signals. If one of the colors is missing from the first sight of the nucleus, skip it.
3. Score only obvious doublets. If inconclusive, look carefully for other single signals in other parts of nucleus. If still not sure and no single signals seen, skip it. If other single signals seen, count as "no doublet".
4. Do not count in areas of high background or debris on the slide, or in areas where nuclei are clumped and obscure the individual nuclear borders.

PROBLEMS WITH COUNTING:

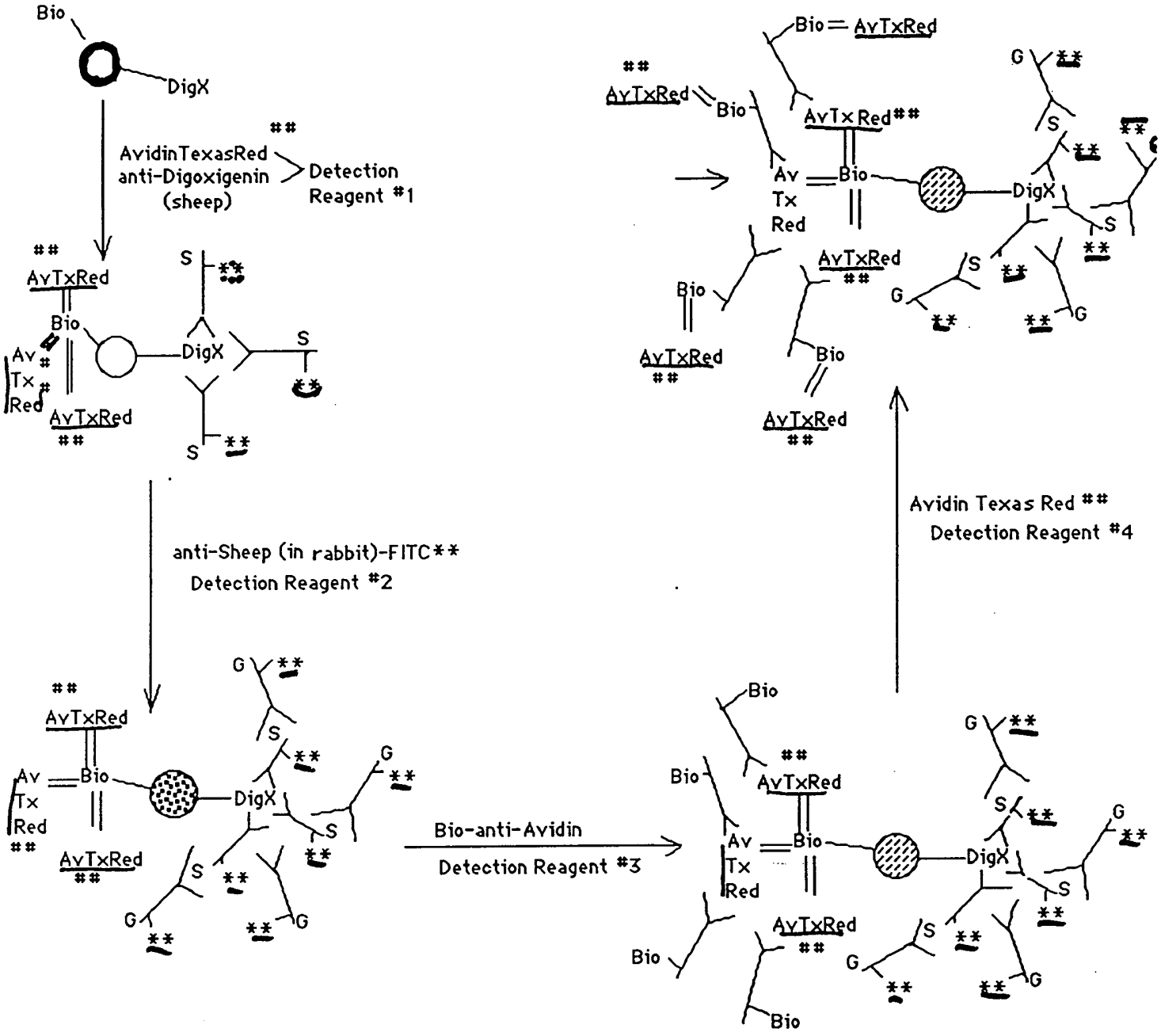
1. Can only count one or two nuclei in a field at a time before the red fades, so in fields with lots of nuclei, only a few get counted. May circumvent this somewhat by partially closing down the first diaphragm along the tube, just outside the lamp housing.
2. Difficult sometimes to define what is a doublet: how close must be a translocation and how close by chance? More than the diameter of the signal away? Not a constant number! Need to hybridize and count some normals (oops, no negative controls!).
3. Signals in slightly different planes of focus means will miss several if not constantly playing with fine focus.
4. Tiring! Need to set up scope on a more comfortable table!

Took 32 photos, ASA 800, will develop at 800. Exposure times were around 7-10 seconds, tried to expose until the red faded.

* Still need more amplification, more blocking, and better washing.

In Situ Amplification Steps and Reagents

Yaremko



Thurs -

Not in Lab - ACS luncheon

FRI.

Dropped pt slides

Made touch preps of Colon case 90-11690

- tumor
- adenoma
- nl mucosa

2 slides of each fixed in 95% ~~ethanol~~ & small amt glacial acetic acid

rest in 95% alcohol

M. ____

I. Hybridization
II. Colon tissue disagg.
III. Microscope
IV. Call A. Keating
V. Drop rest of patient slides.

I. Hybridization – will do following cases:

1. Varga, 48° culture (dropped this AM) c̄ PEM/ABL
2. Varga 48° culture " MSB/ABL
   to ✓ results c̄ better cells – last prep was c̄ recently thawed cells

3. SupB13 c̄ MSB/ABL } pos & neg controls
4. ALH normal c̄ MSB/ABL }
5. ALH normal c̄ PEM/ABL – neg control for double

Colon cells:

6. 90-11690 tumor touch prep 95% a/c :
7. 90-11690 nl mucosa 95% a/c : ? no ep
8. ALH normal lg's : control (in case #7 is no good) and control for probe. not hopef for this

Precipitation / reconst. of probes

	DNA PEM	MSB	ABL	MHS.15	pl DNA	ss DNA	KOAc	ETOH	dextr/fo
Pem(2) ABL	~~24~~ ~~12~~	–	~~10~~	–	✓1	✓1	✓2	✓200	10/10
MSB(3) ABL	–	~~56~~	~~46~~	–	✓1.5	✓1	✓5	✓400	15/15
MHS.15(3)	–	–	–	~~180~~	✓1	✓1	✓2	✓200	15/15

(MSB+PEM: 12λ/sl.) (MHS.15 6λ/sl.) (Abl 5λ/sl)

Combine thru ETOH, ½° @-70°, spin, dry.
Reconstitute c̄ dextran/formamide +70° x 5 min, then 37°.

Probes used: MSB - DigX
PEM12 · DigX
Abl - bio
MH 5.15

Steps:

① Warmer 65° x 4°
② RNAse x1° 37°
③ Wash 2XSSC x4 2'
④ dehydrate + dry
⑤ 70% formamide 70° x2'
⑥ Dehydrate + dry
⑦ Proteinase K 7.5' 37°
⑧ Dehydrate + dry
⑨ Hybridize + seal
⑩ Denature 90° x2', O/N 37°. → 3PM

II. Colon tissue: acquired 1 more case colon tissue -
① 90-11726 (Gray, Arlene) Lav ant. res - colon ca.
fungating centrally ulcerated polypoid tumor.
Touch preps made of tumor → into 95% alc. alone
Normal mucosa into MEM ē pen/strep, refrigerated several hours. Transfered to 4% paraformaldehyde at.
② 90-11690 nl mucosa fresh in fridge over weekend.
Cut off strips of mucosa, minced it as fine as possible in MEM. Divided specimen in half:
a. ½ of cells: 4 ml MEM + 0.5 ml collagenase 200 u/m, into 25 ml flask: into incubator o/n.
b. ½ of cells: transferred to paraformaldehyde. Will do a collagenase digestion p o/n fixation.

Paraformaldehyde prep per Manuelidis
1. 1.6 gm paraformaldehyde into 20 ml H_2O → heat 60° to
2. add equal vol. buffer - I used NaH_2PO_4 (pH 6), 0.2 M
3. adjusted pH ē NaOH to 6.8 - 7.2 (6.93)

III. Microscope: Zeiss rep. not in office - will try tomorrow
IV Informed Dr Listing of unsuccessful results on 1st try ē CML colo

TUES. LY

I. Detection steps, yesterday's run.
II. Begin new hybridization run w/ PEM/ABL
III. Colon tumor disaggregation, cont'd.

I. Detection steps:
- A. Remove coverslips, wash X3 50% formd. at 40 C.
- B. Wash X3 for 3 min, 4X SSC, 40C.
- C. Blocking: used 50/50 5% BSA/SSA.
- D. Detection/amplification: see diagram page.

II. New hybridization run. Will use following slides:

1. CML colony slide W-150, Day 0, 7,8
2. CML colony slide W-150, Day 0, 9.10.
3. BV 173, pos. control.
4. ALH 8/13, neg. control.
5. Vargos, ALL BM
6. Lord, ALL PB
7. J Smith, ALL
8. Orzell, ALL

} All to be done with Pem/ABL.

DNA preparation: Probes used, PEM-12-digX ; - all used
ABL-biotin

	PEM12	ABL	placDNA	ssDNA	KOAC	EtOH	Dex/Form
#1	✓44	✓20	✓4	✓4	✓7	500	20/20
#2	✓44	✓20	✓4	✓4	✓7	500	20/20

Combine, -70 X30 min, spin, dry, reconstitute.
+70 X5 min, then 37 until use.

Slide prep: → all slides EXCEPT ALH neg control were on x 4°

- ✓1. Slide warmer 65 C X4 hr.
- ✓2. RNAse X1 hr, 37 C.
- ✓3. Wash 2X SSC X4 2 min.
- ✓4. Dehydrate & dry.
- ✓5. 70% formd. 70 C X2min.
- ✓6. Dehydrate & dry.
- ✓7. Proteinase K 37 C 7.5 min.
- ✓8. Dehydrate & dry.
- ✓9. Apply probe & seal.
- ✓10. 90 C x2 min, then 37 C o/n.

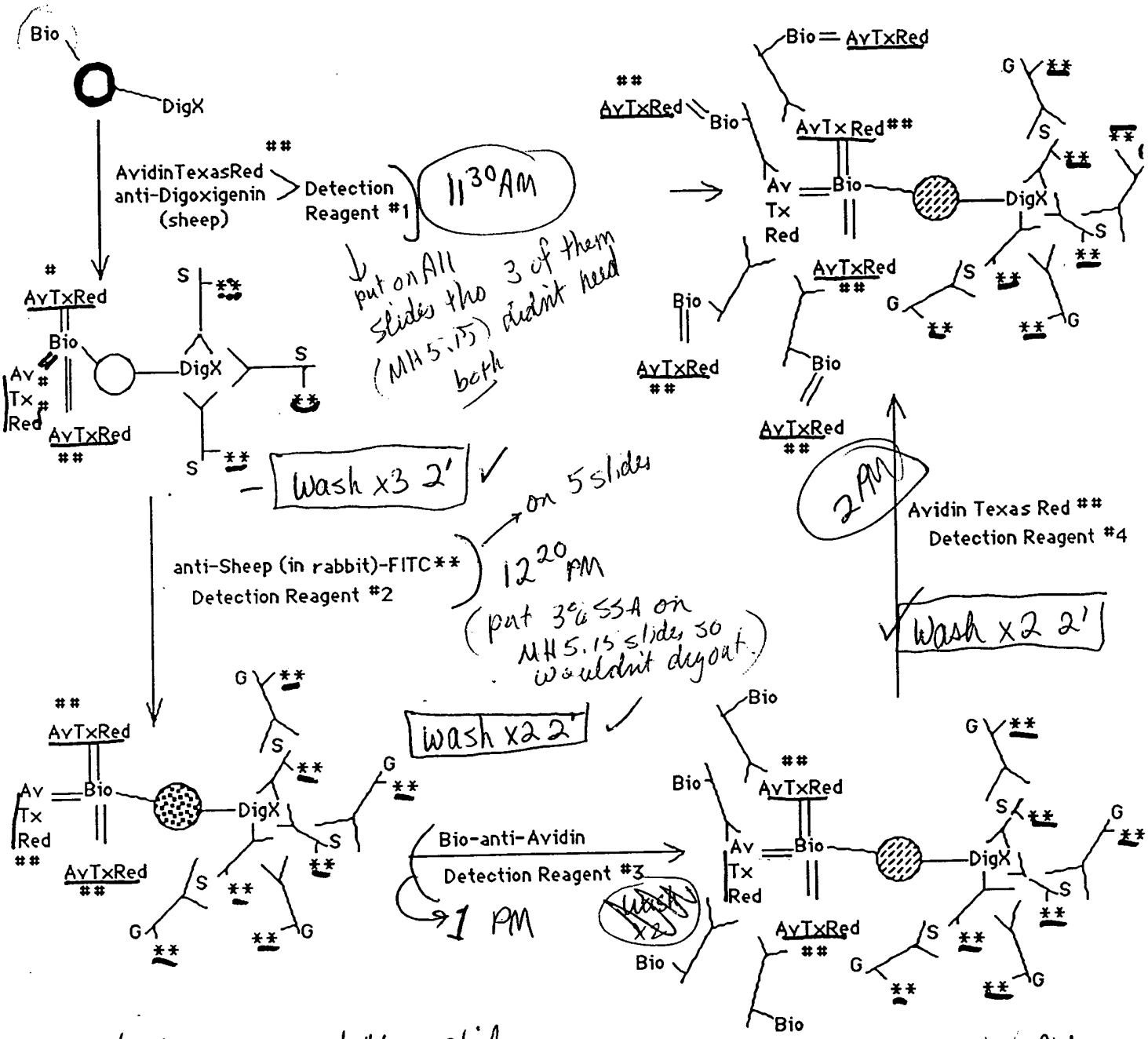
III. Colon tumor disaggregation:

cont'd incubation ā collagenase.
Will seive in AM, pass into PBS, ? trypsinize?

Ly

In Situ Amplification Steps and Reagents

Yaremko



Detection reagent #1: 8 slides

✓ 1200 µl 4x SSC/0.1% Triton
✓ 600 µl 3% SSA
✓ 20 µl TxRAvidin
✓ 20 µl α DigX

Detection reagent #2 for 8 slides

✓ 1200 µl 4x/trit
✓ 600 µl 3% SSA
✓ 20 µl α sheep-FITC.

5

Do not put MH5.15 sl

Detection reagent #3 for 8 slides

1200 µl 4x/trit
600 µl 3% SSA
20 µl Bio αAv.

over

Detection reagent #4

✓1200 λ 4x/trit.
✓600 λ 3%SSA
✓20 λ AvTxR

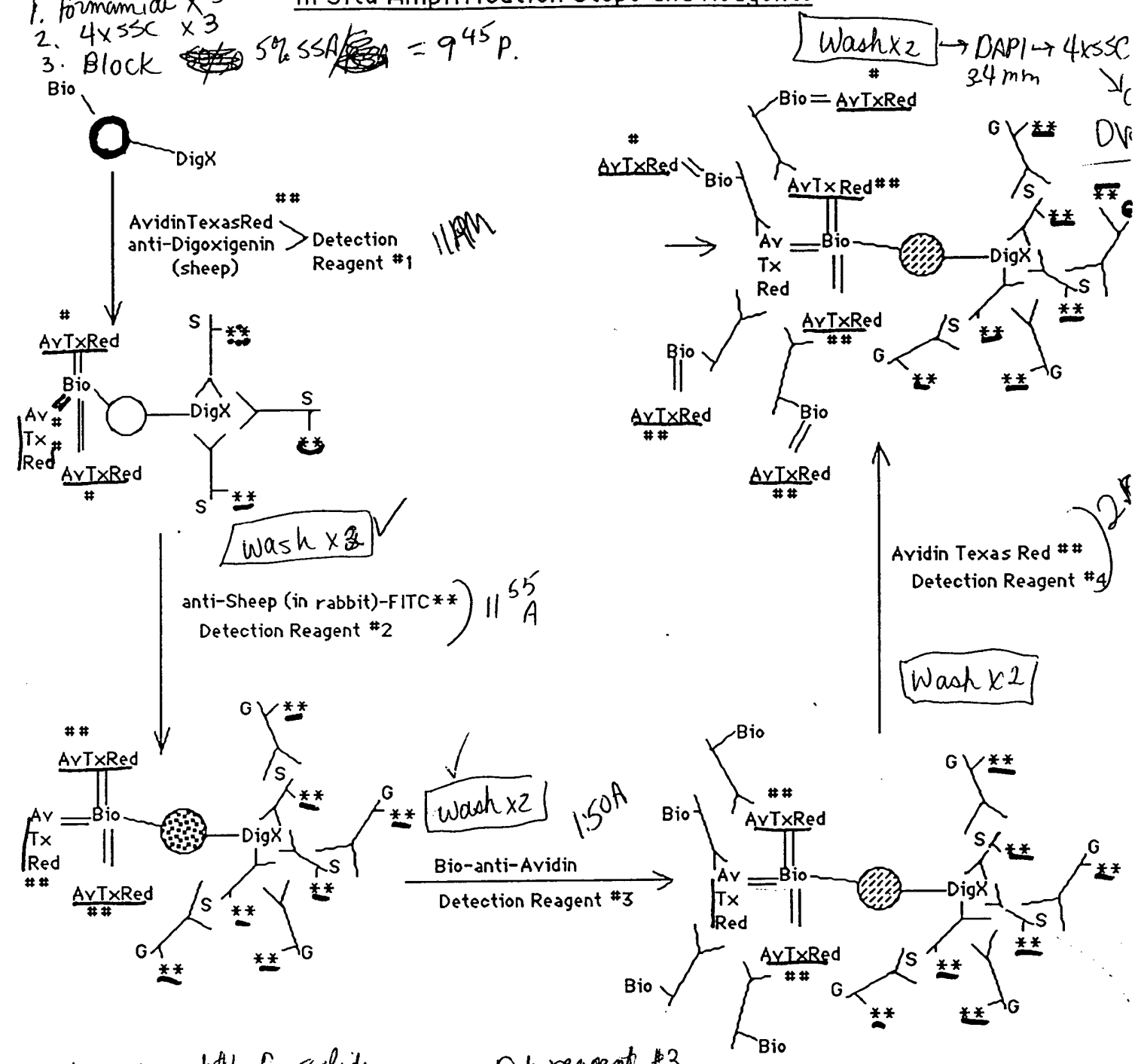
Will examine + photograph Thurs. AM
ā the results of tomorrow's cases.

For Weds

1. Detection steps on slides
2. Colon disaggr.
3. ? review heating slides?
4. ✓ for fax sheets from Zeiss rep.

In Situ Amplification Steps and Reagents

1. formamide X 3
2. 4xSSC x 3
3. Block ~~5% SSA~~ = 9^45 P.



Detection reagent #1 for 8slides
1200 λ 4x/Triton
600 λ SSA
20 λ Av TxR
20 λ α DigY Fitc

Det. reagent #2 for 8sl.
1200 4x/trit
600 RSA
20 α sheep

Det. reagent #3
1200 4xSSC
600 SSA
20 λ α Avidin

Det reagent 4
1200 4xSSC
600 SSA
20 λ Av Tx Red

- Completed at 3PM.

- JSm PEM/SBL slide cracked when plotted mounted & taped it to a 2nd slide — may not be able to view it.

LY

...nurs.

I. Look at slides from last two runs.
II. Start hybridization on same pts. as before, MSB/ABL
III. Nick-translate more probes.
IV. Order materials for colon disaggregation and rhodamine 600.

I. Slide: !!!Background very bad! In all slides except colon tumor slides, a fine green "snowstorm" of nonspecific fluorescence. Probably because of reconstitution of anti-digX (rabbit) in sheep rather than rabbit by mistake. Otherwise probes worked well, but slides of leukemia pt. and cell lines not evaluable because of background.

Colon tumor slides with not enough penetration of probes, and not enough single cells. Will try these again with stronger and longer proteinase K digestion.

II. New hybridization run. Will use following slides:

will not do today – will do c̄ PEM/ABL run Mon. ←

1. ~~CML colony slide W-150, Day 0, 11,12~~
2. ~~CML colony slide W-150, Day 0, 17,18~~
3. SUPB13, pos. control.
4. ALH 8/13, neg. control.
5. Vargos, ALL BM
6. Lord, ALL PB
7. J Smith, ALL
8. Orzell, ALL

~~1&2 to be done with PEM/ABL.~~

3-8 to be done with MSB/ABL.

DNA preparation: Probes used, MSB-digX 10/1/90: 10 ul/slide
ABL-biotin 10/1/90: 5ul/slide
~~PEM12-DigX 9/18/90: 10 ul/slide~~

	MSB1	~~PEM~~	ABL	placDNA	ssDNA	KOAC	EtOH	Dex/Form
~~P/A~~	--	~~20~~	~~10~~	~~4~~	~~4~~	~~7~~	~~500~~	~~20/20~~
M/A	~~60~~ 72 (12/slide)	-	30	6	6	10	300	30/30

ERROR

Combine, -70 X30 min, spin, dry, reconstitute.
+70 X5 min, then 37 until use.

Slide prep:

1. ✓ Slide warmer 65 C X4 hr.
2. ✓ RNAse X1 hr, 37 C.
3. ✓ Wash 2X SSC X4 2 min.
4. (circled) Dehydrate & dry.
5. 70% formd. 70 C X2min.
6. Dehydrate & dry.
7. Proteinase K 37 C 7.5 min.
8. Dehydrate & dry.
9. Apply probe & seal.
10. 90 C x2 min, then 37 C o/n.

Put in (... of ssD... plac... Too much supp... S...

Not enough probe to cont., so will do tomorrow
(... stopped slides at step 4)

14 cont'd

III. Nick translate.

	Exp.DNA	RxnB	dNTP	DigX-UTP or bio-UTP	H2O	DNase	DNApol.
Abl	4	5	5	5 (bio)	23	4	4
MSB	6	5	5	5 (DigX)	21	4	4
Pem	4	5	5	5 (DigX)	23	4	4

DNase dilution 1 in 9= 1:10
1 of 1;10 into 9 = 1:100
3 of 1:100 into 12 = 1:500.

Combined all but DNA pol. Reaction started: 2:23P.
DNA pol added at: 3:19P.
Reaction stopped w/ 5 ul 200mM EDTA, 10' at 65 C.
Will run on minigel in A.M.

IV. Order supplies , 2 of each:

1. Sigma D5527 Dulbecco's PBS 500ml $8.50
2. Sigma H 8389 CMF-Hank's BSS 500ml $9.25
3. Vector: Rhodamine-600-Avidin D A-2005 $50 5 mg.

Make: 4x SSC

4x SSC/0.1% Triton

3% Rabbit serum

5% SSA

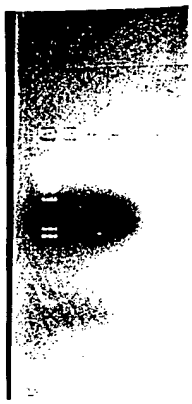
RNAse soln.

In AM

1. Screen all slides – discard all bad ones
   Keep good ones.
2. Run gel
3. complete hybn. if probes OK

LY

I. Ran gel– first one λ standard
then ABL
MSB
PEM



No cutting at all!
So, did not complete ISH run.
Amounts, however, seem OK. (4, Abl) (6, MSB) (4, PEM 12)
Will proceed as follows:

Mon: Another nick translation reaction –
1. DNAse 1°
2. DNA pol + DNAse 1°
3. DNA pol 1°

Run gel: if OK, gene clean + prepare for a run Mon. + Tues. with all slides.

II. Colon disaggreg. – 1) working out steps of disaggr. from Jakoby + Pastan, cell culture, (see printouts)

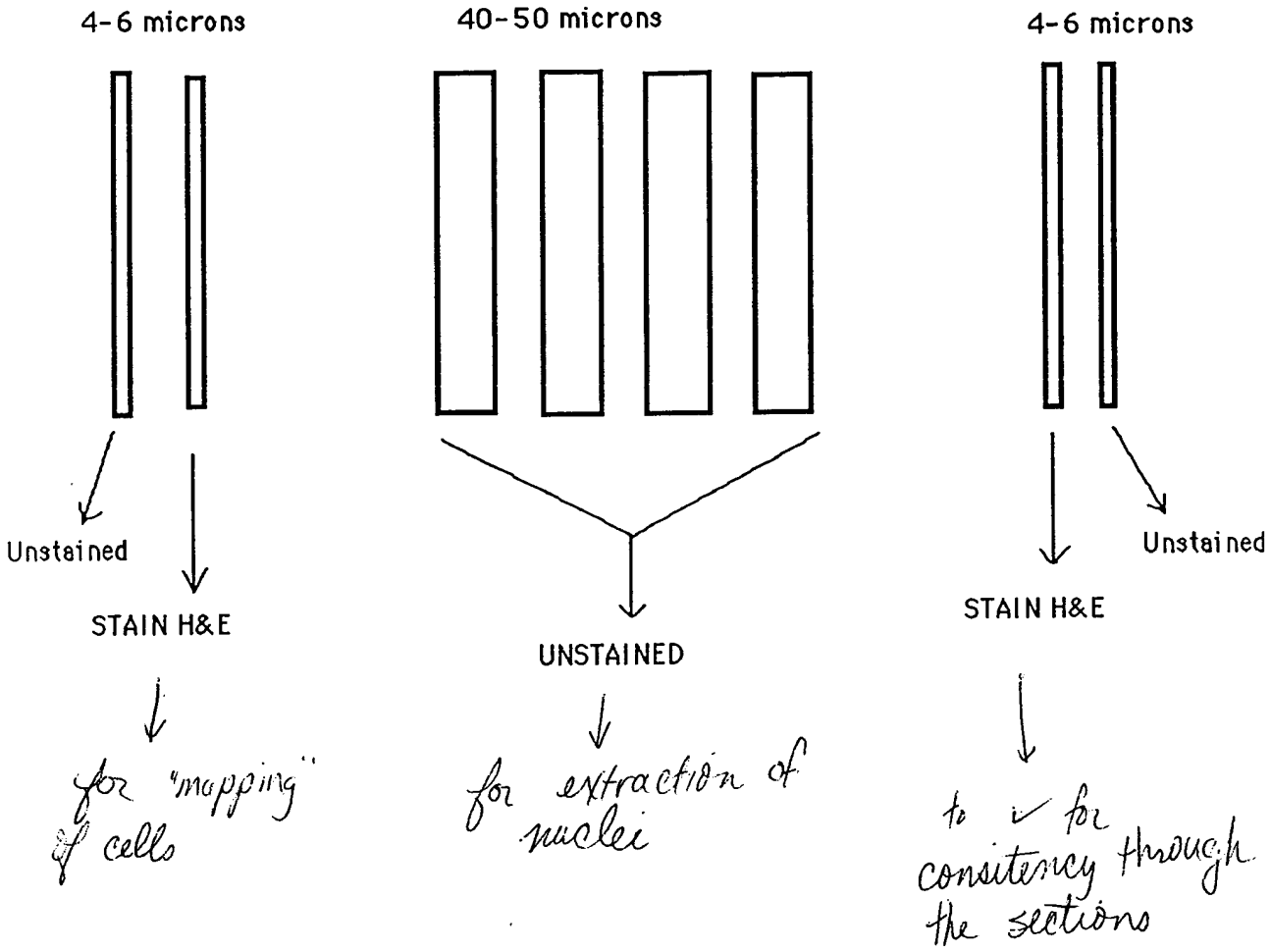
2) Asked T. Montag to have blocks cut from recent colon ca case ... as per accompanying diagram. Will include these cells in runs ⓒ fresh and briefly fixed tissue.

3) Materials ordered.

LY

90-11690 COLON TUMOR AND ADENOMA

Blocks number: C, E, G.



Will save unstained slides – ? factor VIII stains to look for vessel in ? tumor

[redacted]LY

I. Nick translate
II. Make enzyme solutions and paraformaldehyde, Tris quencher
III. Pick up slides from T. Montag
IV. Acquire tissue from pathology.

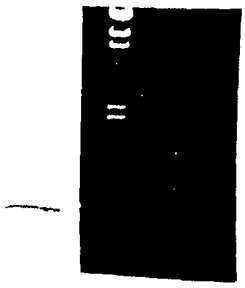
I. Nick translate.

	Exp.DNA	RxnB	dNTP	DigX-UTP or bio-UTP	H2O	DNase	DNApol.
Abl	4	5	5	5 (bio)	23	4	4
MSB	6	5	5	5 (DigX)	21	4	4
Pem	4	5	5	5 (DigX)	23	4	4

DNase dilution 1 in 9= 1:10
1 of 1:10 into 9 = 1:100
3 of 1:100 into 12 = 1:500.

Combined all but DNA pol. Reaction started: 8:35 AM
DNA pol added at: 9[35]A
DNAse stopped w/ 10' at 65 C at: 10[35]A
DNA pol. cont'd x1 hr, stopped w/ 5 ul 200mM EDTA at: 11[35]A

Ran gel:



λ - Abl - MsB - Pem12) labelled
all ☺ good cutting.

Success! So repeated reaction with more Pem12 & MSB 1:

Nick translate.

	Exp.DNA	RxnB	dNTP	DigX-dUTP	H2O	DNase	DNApol.
MSB	6	5	5	5 (DigX)	21	4	4
Pem	4	5	5	5 (DigX)	23	4	4

*(prepared 2 reaction tubes of each)

DNase dilution 1 in 9= 1:10
1 of 1:10 into 9 = 1:100
4 of 1:100 into 16 = 1:500.

(cont'd)

Combined all but DNA pol. Reaction started: 1:50 PM
DNA pol added at: 2 50 P
DNAse stopped w/ 10' at 65 C at: 3 50 PM
DNA pol. cont'd x1 hr, stopped w/ 5 ul 200mM EDTA at: 4 50 PM

Ran gel:

All OK. will Gene clean.
Amts?

PEM 12 into 120+ 100λ of TE
MSB into 75λ each
↓
Combined, fo
150λ MSB
220λ PEM

Labeled 10/25

II.
Made 500 cc 0.1 M Tris pH 7.2
50 ml 1M Tris + 450 H_2O
Autoclave in AM.

Will make rest of exp solns in AM.
as well as 3+4.

Called Rush immunology, asked for copy of disaggreg. protocols for paraffin sections

T: LY I. Hybridization run
II. Paraffin tissue disaggregation protocol
III. Prepare enzyme solutions

I. Hybridization run. Will use following slides:

1. CML colony slide W-150, Day 0, 11,12
2. CML colony slide W-150, Day 0, 17,18
3. BV173, pos. control.
4. ALH 8/13, neg. control.
5. Vargos, ALL BM x2
6. Lord, ALL PB x7
7. J Smith, ALL x 2
8. Orzell, ALL x 2
9. SUPB13, pos. control.
10. ALH neg. control.

1-4 to be done with PEM/ABL.

9-10 to be done with MSB/ABL.

5-8 w/ both.

Total of 14 slides. 8 for PEM/ABL, 6 for MSB/ABL.

DNA preparation: Probes used, MSB-digX 10/22/90: 12 ul/slide
ABL-biotin 10/22/90: 5ul/slide
PEM12-DigX 9/22/90: 12 ul/slide — all used!

	MSB1	PEM	ABL	placDNA	ssDNA	KOAC	EtOH	Dex/Form
P/A	--	94	40	8	8	15	500	40/40
M/A	72	-	30	8 6	8 6	11	500	30/30

Combine, -70 X30 min, spin, dry, reconstitute.
+70 X5 min, then 37 until use.

Slide prep:

1. Slide warmer 65 C X4 hr.
2. RNAse X1 hr, 37 C.
3. Wash 2X SSC X4 2 min.
4. Dehydrate & dry.
5. 70% formd. 70 C X2min.
6. Dehydrate & dry.
7. Proteinase K 37 C 7.5 min.
8. Dehydrate & dry.
9. Apply probe & seal.
10. 90 C x2 min, then 37 C o/n.

II. Tissue disaggregation.

Over.

LY

Tissue Disaggs

Tissue Disaggs: Rec'd nl colon mucosa case 90-12079. pt # 1956909

Divided & minced into

fresh 4% paraformald. fixed 2°

↓

0.1 M TRIS x1°

↓ spun

PBS c sodium azide

(collagenase trypsin mix x45'

Trypsin alone)

Mixed small amt 0.01-0.03g trypsin c 4ml BSS + 1ml aliquot collagenase (2000u/ml)

inc. 45' 37°

↓

Trypsin protocol

↓

into PBS

For AM.

Trypsin Soln.

CMF - BSS. Kept at 37° while mixed & filtered trypsin/co

↓

added double vol. FCS — refrigerated O/N.

Weds

↓

Trypsin soln. disaggregg. per protocol.

one tube BSS/FCS

P Fix paraformald. Tris PBS.

P Fix hypotonic KCl 3:1 fix

LY

I. Detection steps for hybridizaton run.
II. Cont. disaggregation protocol on fixed tissue.
III. Order pepsin & materials for paraffin disagg.

III. Order supplies.

*1. Sigma D5527 Dulbecco's PBS 500ml $8.50 → 4
* 2. Sigma H 8389 CMF-Hank's BSS 500ml $9.25 → 2
3. Sigma: Trypsin inhibitor T 6522 100 mg. $27.15 → 1
4. Sigma: Pepsin 1:60,000 P7102 5g $49.30 → 1
5. Sigma Collagenase C 9407 100mg $31.60 → 1
6. Sigma Cell dissociation kit CD-1 $69.50 → 1

II. Summary of disaggregation steps:

Normal mucosa, minced into 2-3mm pieces.

Fixed in fresh 4% paraformaldehyde for 2 hr. room temp.
↓
Quench with 0.1 M Tris X1 hr.
↓
Wash PBS X3.
↓
Trypsin soln. (37 C) and pipetting several times.
↓
Wash PBS X3.
↓
① Fixed, trypsin alone
② Fixed, combo enz. tx.

4 tubes of disaggs. cells

Placed in CMF-BSS
Kept at 37 C til enz. ready
↓
Incubated with combined collagenase/trypsin soln. 45 min, 37 C.
↓
Added double volume FCS.
↓
Trypsin soln. alone (37 C)
Several rounds of pipetting.
↓
Spin, divided pellet.
↓
Fixed in fresh 4% paraform.
Quench in Tris.
Reconstitute in PBS.
↓
③ Fresh, combo, post-fix paraform.

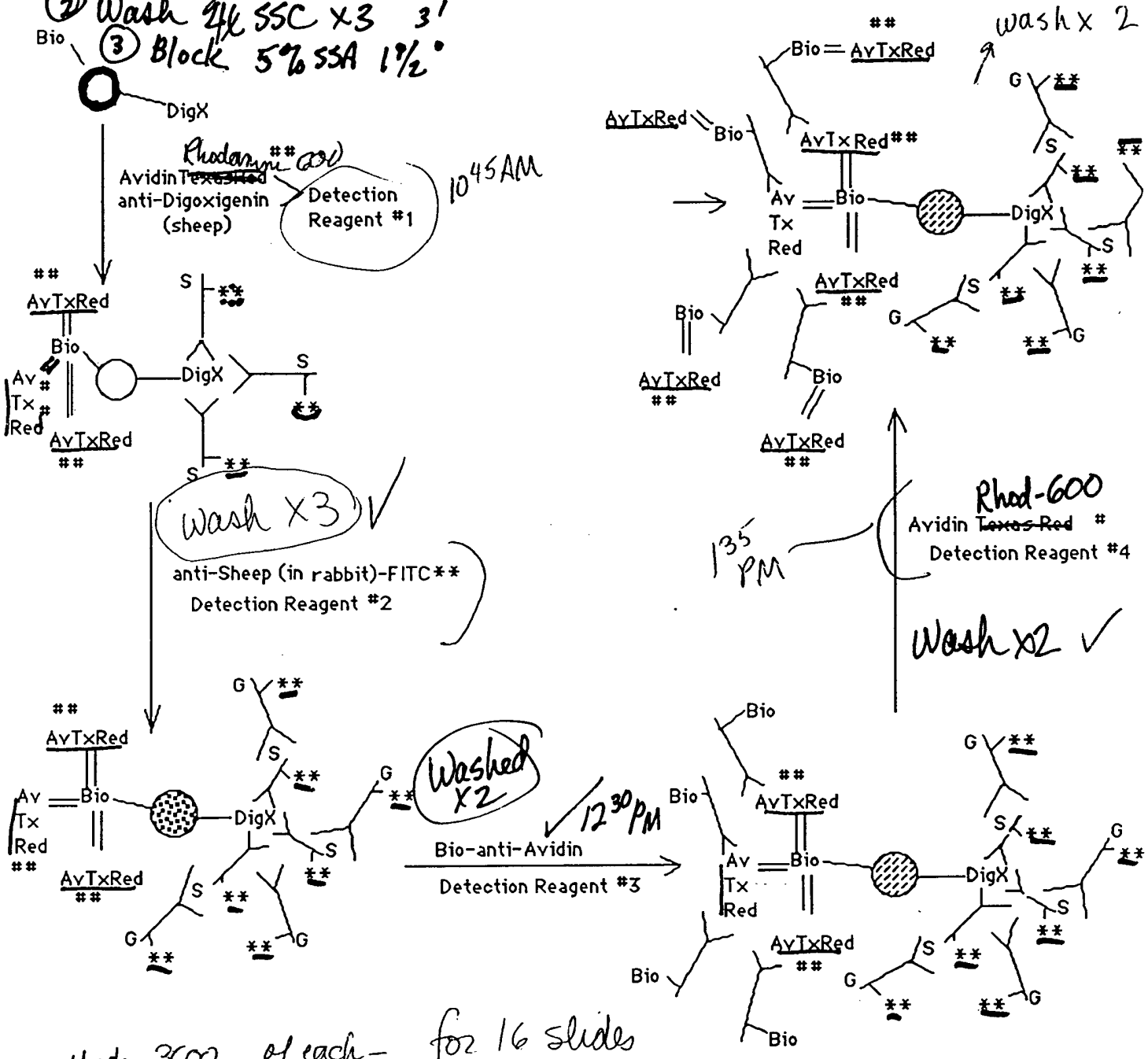
Stored 4 C.
Stored! add'l treat

Hypotonic KCl 3:1 fix.
↓
④ Fresh, combo, post-fix 3:1.
↓
very few cells!

In Situ Amplification Steps and Reagents

Yaremko

1. Formamide 50% x3 5'
2. Wash 4x SSC x3 3'
3. Block 5% SSA 1½'



Made 3600 of each — for 16 slides

- 12000 SSA or RSA
- 2400 4xSSC/0.1% Triton
- 38 x of each fluorochrome

P amplification & washes, DAPI x 3-5'

Fast wash 4xSSC

DABCO & coverslip. Will examine in AM.

T: I. Evaluate colon disagg. samples.
II. Hybridize colon sample if O.K.
III. Collect more colon: Tumor + normal.
IV. Evaluate slides from last run.

I. Colon samples:

A. Fixed, trypsin alone: good dispersion of cells, but still many clumps. Cytoplasm still largely intact. Nuclear detail good!

B. Fixed, combo collagenase/trypsin followed by trypsin: good dispersion of cell, better than trypsin alone. Cytoplasm often stripped from cells. Nuclear detail remains good.

C. Fresh, combo treatment, post-fixed in 3:1 fix: No cells.

D. Fresh, combo treatment, post-fixed paraformaldehyde: Cells destroyed, poor detail, poor staining, but disaggregated.

Conclusion: Fixed cells give better preservation with adequate dispersion. Will hybridize some from both the fixed preps to compare.

II. Hybridization: Eight slides to be done with MH5.15 ~~biotin~~. Dig X.

1. Fixed trypsin alone with glue solution on slides.
2. ""
3. Fixed trypsin alone, no glue.
4. Fixed, combo enzyme treatment, with glue-treated slides
5. "" " "
6. Fixed combo treatment, no glue.
7. Touch prep, 90-11760, 95% alcohol fixed.
8. JK control normal lymphocytes.

MH5.15	Plac. DNA	ssDNA	KoAC	EtOH	Dextr/ Formam.
64	8	8	7	300	40/40

Combine, -70 X30 min, spin. Reconstitute, 5 min at 70 C. 37 C til use.

Slide prep:

1. Slide warmer 65 C X3 hr.
2. RNAse X1 hr, 37 C.
3. Wash 2X SSC X4 2 min.
4. Dehydrate & dry.
5. 70% formd. 70 C X2min.
6. Dehydrate & dry.
7. Proteinase K 37 C 7.5 min.
8. Dehydrate & dry.
9. Apply probe & seal.
10. 90 C x2 min, then 37 C o/n.

III. Collect more colon: two resections on schedule today